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Multiple Promoter Targeting Sequences exist in *Abdominal-B* to regulate long-range gene activation

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Abstract

In complex genomes, insulators set up chromatin domain boundaries and protect promoters from inappropriate activation by enhancers from neighboring genes. The *Drosophila Abdominal-B* locus uses insulator elements to organize its large regulatory region into several body segment-specific chromatin domains. This organization leads to a problem in enhancer–promoter communication, that is, how do distal enhancers activate the *Abd-B* promoter when there are several insulators in between? This issue is partially resolved by the Promoter Targeting Sequence, which can overcome the enhancer blocking effect of an insulator. In this study, we describe a new Promoter Targeting Sequence, PTS-6, from the *Abd-B* 3′ regulatory region. PTS-6, comprised of approximately 200 bp, was found to bypass both homologous *Abdominal-B* insulators, such as *Fab-7* and *Fab-8*, and a heterologous insulator, *suHw*. Most importantly, it also overcomes a combination of two insulators such as *Fab-7/Fab-8*. Thus, PTS-6 could, in principle, target remote enhancers that are separated from the *Abd-B* promoter by multiple insulators. In addition, PTS-6 selectively targets the distal enhancer to only one transgenic promoter, and it strongly facilitates *Abd-B* enhancers. These results suggest that promoter targeting is necessary for long-range enhancer–promoter communication in *Abd-B*, and PTS elements could be a common occurrence in large, complex genetic loci.

Keywords: PTS; PTS-6; *Abd-B*; *Fab-7*; *Fab-8*

Introduction

In higher eukaryotes, developmentally regulated genes often contain a large number of transcriptional enhancers that are located many kilobases away from the promoter. The *Drosophila Bithorax* gene complex (BX-C), which controls the body plan along the anterior–posterior axis (in the posterior of the embryo), is comprised of more than 300 kb of DNA. Yet, it encodes only three homeotic genes, *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*), and *Abdominal-B* (*Abd-B*) (Lewis, 1978; Martin et al., 1995; McGinnis and Krumlauf, 1992; Morata et al., 1986). Each of these genes contains a large complex regulatory region that is organized into body Parasegment (PS)-specific domains. *Abd-B* contains five such domains, *infraabdominal-5* (*iab-5*), *iab-6*, *iab-7*, *iab-8*, and *iab-9*, which function in PS10, 11, 12, 13, and 14, or roughly the 5th through the 9th abdominal segments (Boulet et al., 1991; Celniker et al., 1989; Duncan, 1987; Karch et al., 1985; Morata et al., 1986; Sanchez-Herrero et al., 1985). Each of these *iab* domains is believed to contain enough *cis*-regulatory information to control *Abd-B* in a specific abdominal segment. Several studies have led to the identification of three early embryonic enhancers, IAB5, IAB7, and IAB8, from these regions (Barges et al., 2000; Busturia and Bienz, 1993; Zhou et al., 1999). The *Abd-B* most 3′ IAB5 enhancer is positioned at equal distances, more than 50 kb away, from both *abd-A* and *Abd-B* promoters, yet, it normally activates the latter (Martin et al., 1995). Thus, a central question regarding complex genetic loci, such as the BX-C, is how an enhancer element consistently finds the right promoter.

Specialized DNA elements, other than enhancers and promoters, have been found in the BX-C to either regulate the activity of tissue-specific enhancers or to modulate long-range enhancer–promoter communications. For example, *Polycomb* and/or *Trithorax* Response Elements (PRE/TREs)

Abbreviations: PTS, Promoter Targeting Sequence; *Abd-B*, *abdominal-B*; *Fab-7*, *Frontabdominal-7*; *iab*, *infraabdominal*.

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(Barges et al., 2000; Busturia and Bienz, 1993; Busturia et al., 1997; Chan et al., 1994; Hagstrom et al., 1997; Muller et al., 1999; Zhou et al., 1999) have been found to recruit protein complexes containing products from the *Polycomb* and *Trithorax* group genes to repress or activate the chromatin and, therefore, maintain the activities of early embryonic enhancers. As a result, these enhancers are either "on" or "off" in specific cells during late embryogenesis and throughout adulthood (Paro et al., 1998; Pirrotta, 1998; Pirrotta et al., 2003).

Chromatin boundary elements, such as *Miscadastral Pigmentation* (*MCP*) (Karch et al., 1994), *Frontabdominal-7* (*Fab-7*) (Hagstrom et al., 1996; Karch et al., 1994; Mihaly et al., 1997; Zhou et al., 1996), or *Fab-8* (Barges et al., 2000; Zhou et al., 1999), are also found in the *Abd-B* locus (see Fig. 1). A boundary element, also known as an insulator, usually has two activities; first, it provides barrier function to prevent the spreading of silencing activities such as the formation of heterochromatin. This activity is responsible for protecting transgenes from position effect variegation (PEV) due to their insertion near heterochromatin. Insulators also exhibit enhancer blocking activity by preventing transcription activation when inserted between an enhancer and a promoter. Insulators have been discovered in species from yeast to humans; notable examples include the human *Igf2/H19* imprinting control region (Hark et al., 2000; Kanduri et al., 2000), the chicken *β-globin HS4* element (Bell et al., 1999; Chung et al., 1993), the *Drosophila suppressor of hairy wing* (*suHw*) from the *gypsy* insulator (Dorsett, 1993; Geyer and Corces, 1992), and the *scs/scs'* elements from the *Drosophila hsp70* locus (Gaszner et al., 1999; Kellum and Schedl, 1992; Udvardy et al., 1985; Zhao et al., 1995).

It has been proposed that the *Fab-7* and *Fab-8* elements function as chromatin domain boundaries in the *Abd-B* locus to restrict chromatin regulatory events, such as the function of PREs/TREs so that each *iab* domain is functionally "isolated" from its neighbors (Mihaly et al., 1998; Vazquez et al., 1993). However, both of the *Fab* elements also block enhancer–promoter interactions when tested in transgenic flies (Barges et al., 2000; Hagstrom et al., 1996; Zhou et al., 1996, 1999). This activity creates a problem for enhancers located within *iab-5*, *iab-6*, and *iab-7* elements because these enhancers must communicate with the *Abd-B* promoter at the appropriate time and in the appropriate segments during development. The *iab* elements must overcome the enhancer blocking activity of the *Fab-7*, *Fab-8*, and other potential insulators in order to activate *Abd-B*. Thus, an additional mechanism(s) is necessary to mediate long-range enhancer–promoter interactions over intervening insulator elements in *Abd-B*.

The recently identified Promoter Targeting Sequence (PTS) may provide insight into such a mechanism (Zhou and Levine, 1999). The PTS has an anti-insulator activity: it allows an enhancer to activate its promoter despite an intervening insulator. The PTS also facilitates long-distance enhancer–promoter interactions and selectively activates a single promoter when two are included in the same transgene (Lin et al., 2003, 2004). These studies support the model that the PTS may mediate long-distance gene activation in *Abd-B* by overcoming intervening insulators such as *Fab-7* and *Fab-8*, facilitating more 3′ enhancers, and specifically activating the

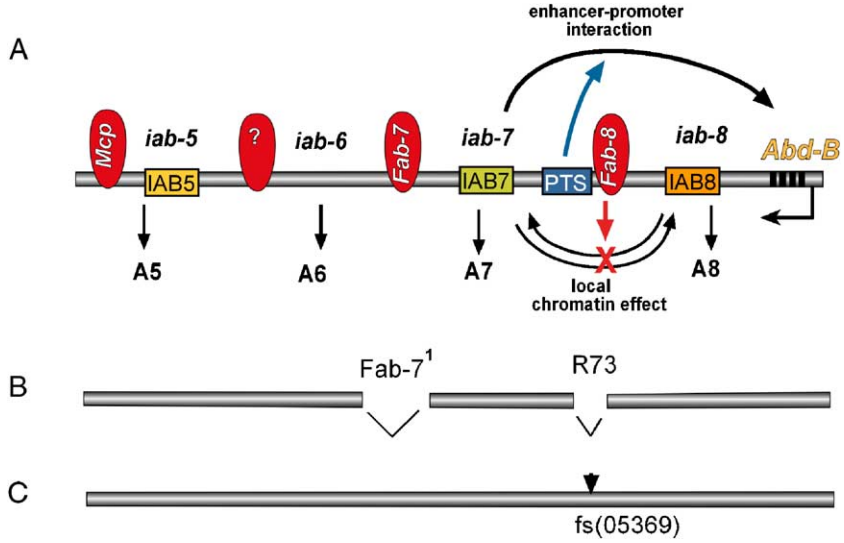

Fig. 1. A summary of *cis*-interactions in the *Abd-B* locus. (A) The *Drosophila Abd-B* locus consists of four 3′ abdominal parasegment (PS)-specific regulatory domains, termed *infraabdominal-5* (*iab-5*), *iab-6*, *iab-7* and *iab-8*, which regulate *Abd-B* function corresponding to ps10, ps11, ps12, and ps13, or roughly abdominal segment A5, A6, A7, and A8, respectively. These domains are separated by boundary elements such as *Frontabdominal-7* (*Fab-7*) and *Fab-8*. In transgenic flies, the *Fab-7* and *Fab-8* elements function similarly to insulator elements and block enhancer–promoter interactions. However, in the endogenous locus, they do not interfere with enhancers such as IAB5 and IAB7, possibly due to the presence of the PTS elements. The PTS possesses an anti-insulator activity and may target these enhancers to the *Abd-B* promoter. In doing so, it converts the *Fab* elements into local chromatin boundary elements. (B) Diagram showing mutant allele $Fab\text{-}7^{R73}$ removing approximately 800 bp DNA from the PTS region and a 3.7 kb DNA from the *Fab-7* region. This mutant exhibits loss of *Abd-B* function in the A5, A6, and A7 segments when hemizygous for this mutation (Zhou and Levine, 1999). (C) A female sterile *P*-element insertion *fs(05369)* into the PTS region. This mutation exhibits mild *Abd-B* loss of function in the 7th abdominal segment (Zhou and Levine, 1999).

Abd-B promoter. Consequently, the PTS converts the *Fab* elements into local chromatin boundary elements to restrict the active or repressed chromatin within each regulatory domain (Fig. 1).

Mutations in the PTS region lead to loss of function of *Abd-B*, supporting the role of PTS in facilitating enhancer–promoter interactions (Zhou and Levine, 1999). A *P*-element insertion mutation in the PTS region leads to a moderate loss of *Abd-B* function in the 7th abdominal segment (Fig. 1). However, when a mutation of PTS is combined with a 3.7 kb deletion in the *Fab-7* boundary region (Fig. 1), a much stronger loss of function is observed: abdominal segments from the 5th through the 7th are transformed into copies of the 4th, suggesting that PTS may be functionally redundant with additional PTS elements removed from the *Fab-7* region (Zhou and Levine, 1999). To test this possibility, we analyzed the DNA near the *Fab-7* boundary for DNA sequences with promoter targeting function. In this paper, we report the identification of a new PTS element, PTS-6, located just next to the *Fab-7* insulator. This element permits an enhancer to selectively activate a transgenic promoter, bypassing the intervening *Fab-7* and other insulators. In addition, it can overcome a combination of two insulators such as *Fab-7* plus *Fab-8*. We found that both PTS elements could overcome multiple insulators and function from a number of positions relative to the enhancer and the insulator. These results strongly support the promoter targeting model of long-distance transcription activation in *Abd-B* and further suggest that multiple PTS elements may work synergistically to regulate enhancer–promoter interactions in *Abd-B*.

Methods

Plasmid constructions

To generate the *P*-transgenes shown in Fig. 2, we inserted either 1.6 kb of λ DNA (HZλN), a 0.8 kb *Fab-7* insulator (HZFN) (Hagstrom et al., 1996; Mihaly et al., 1997; Zhou et al., 1996), or a 3.7 kb *Fab-7* region (Karch et al., 1994) (W170) into the *Bgl*II site downstream of the *Transposase* (*Tp*)-*lacZ* gene of the HZGN vector (Lin et al., 2003). For the *P*-transgenes in Fig. 4, different *Bam*HI–*Bgl*II truncated fragments (position shown in Fig. 4) from the 3.7 kb *Fab-7* boundary (for W263, the DNA fragment was flanked by FRT sites to form a *Bgl*II insert) were inserted into the *Bgl*II site downstream of the *Transponsase* (*Tp*)-*lacZ* gene and *SuHw* insulator of #125 construct, respectively. A 1.6 kb *Pst*I IAB-8 enhancer was inserted into the *Pst*I site of C4PLZ vector to generate the W76 construct. Thereafter, a 0.7 kb *Bam*HI–*Bgl*I I *Fab-8* and a 0.8 kb *Bam*HI–*Bgl*II *Fab-7*, either with or without the 200 bp *Bam*HI–*Bgl*I PTS-6, were sequentially inserted into the *Bam*HI site of a modified *pBluescript* that contains an additional *Not*I site converted from the *Kpn*I site. A *Not*I fragment including *Fab-8* plus *Fab-7*, respectively, with or without PTS-6, was inserted into the *Not*I site between *Tp-lacZ* gene and IAB-8 enhancer of W76 vector to generate W267 and W270. A 1.6 kb *Bam*HI ME fragment (including *Fab-8* and PTS-7) was inserted into the *Bgl*II site of #125 vector in different orientations to generate W114 and W115.

P-element transformation and in situ hybridization

P-element transformation vectors containing *lacZ* and *white* reporter genes were introduced into the *Drosophila* germline by injecting yw^{67} embryos as described previously (Rubin and Spradling, 1982). Between 15 and 35 independent transformant insertions were obtained for each of the recombinant *P*-elements shown. In situ hybridization was performed essentially as described in previous reports (Tautz and Pfeifle, 1989; Zhou and Levine, 1999).

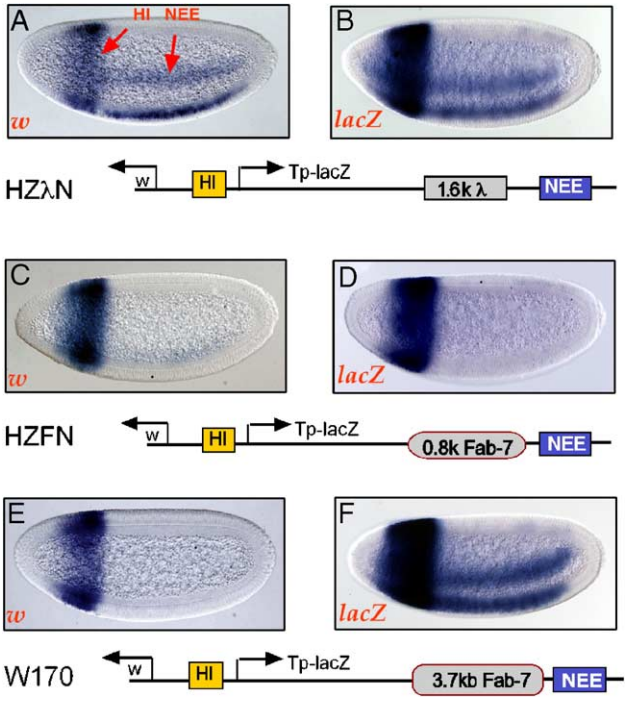

Fig. 2. An anti-insulator activity exists within the 3.7 kb *Fab-7* region. Transgenic embryos carrying different transgenes were hybridized with Digoxigenin-labeled antisense RNA to either the *white* or the *lacZ* gene and were stained with alkaline phosphatase conjugated anti-Digoxigenin antibody (Roche) followed by reactions in NBT/BCIP solution (Tautz and Pfeifle, 1989). Processed embryos were mounted on glass slides. (A) Staining for *w* expression. Control constructs with a spacer inserted between *lacZ* and the 3′ located NEE. The HI enhancer activates transcription in the anterior region of the embryo, while NEE activates *w* in the lateral region (arrows). (B) Staining for *lacZ* expression. Same construct as in panel (A). (C) *w* expression is activated by HI, but not, or minimally, by NEE when the 0.8 kb *Fab-7* is inserted at the 3′ position between *lacZ* and NEE. (D) Similar to *w*, *lacZ* is activated by HI but not NEE. Staining represents most embryos, which show no NEE activity. (E) In the line shown, *w* is activated by HI only. (F) Instead of blocking NEE, the 3.7 kb DNA from *Fab-7* boundary region (include *Fab-7*) actually facilitates the NEE–*lacZ* interaction. Compare with panel (B).

Fly strains and crosses

Transgenic flies expressing the Flip recombinase were kindly provided by Gary Struhl and Steve Small (Wu et al., 1998). To recombine different FRT-flanked DNA elements away from the transgene, females carrying the transgene were mated with males that express the *Flp* recombinase under the control of a sperm-specific *tubulin* promoter (Wu et al., 1998). In F_1 males, the recombinase binds the FRT sites and deletes the intervening DNA. These male flies were collected and mated to *yw* virgin females to establish stocks that were subsequently analyzed by RNA in situ hybridization.

Results

Previous studies have demonstrated that the PTS overcomes the enhancer blocking activity of an insulator and selectively targets and facilitates a distal enhancer to one of

two transgenic promoters (Lin et al., 2003; Zhou and Levine, 1999). In addition, the promoter targeting function is strain-specific: when a collection of individual strains is examined, the enhancer only activates the proximal promoter in a portion of the strains (Type I strains). In other strains, this enhancer only activates the distal promoter (Type II strains). In the remaining strains, the enhancer is blocked by the insulator, and neither promoter is activated (Type III strains) (Lin et al., 2004).

Identification of a new promoter targeting activity from the 3′ Abd-B

Domain boundary regions of the *Abd-B* locus appear to have multiple *cis*-regulatory elements with similar organizations. For example, both *Fab-7* and *Fab-8* are comprised of an insulator located 3′ of a PRE element (Mihaly et al., 1998). It is possible that other *cis* elements, such as the PTS, are also similarly arranged. Because the original PTS is located just 3′ to the *Fab-8* insulator, the best chance of locating a new PTS element will be the region 3′ to the *Fab-7* insulator. For this reason, we tested genomic DNA near *Fab-7* for potential anti-insulator activity. To detect this activity, we analyzed a 3.7 kb *Hin*dIII fragment encompassing the *Fab-7* region, in a transgenic *P*-element, shown in Fig. 2. This region contains the 0.8 kb *Fab-7* element and a nearby 5′ PRE (Hagstrom et al., 1996, 1997; Mihaly et al., 1997; Zhou et al., 1996). We reasoned that, if the 3.7 kb DNA contains a PTS, it should overcome *Fab-7* and target a distal *rhomboid* neuroectoderm enhancer (NEE) (Ip et al., 1992) to one of the 5′ promoters.

As shown in Figs. 2A and B, when placed at 1.6 kb away from the 3′ end of *lacZ*, the NEE enhancer directs transcriptional activation of both *w* and *lacZ* genes, producing ventral lateral stripes along the anterior–posterior axis of the embryos. When the 0.8 kb *Fab-7* was inserted between the 3′ end of *lacZ* and the more distal enhancer, NEE activity is either severely attenuated or totally abolished (Figs. 2C, D). This result is consistent with our earlier observation (Zhou et al., 1996). However, when the 3.7 kb DNA from the *Fab-7* genomic region was inserted in this location, the NEE enhancer is not always blocked in a number of transgenic lines examined. Instead, NEE selectively activates either the *lacZ* promoter (Figs. 2E, F) or the *w* promoter (not shown) in a strain-specific manner. A total of 19 transgenic strains were analyzed, and four exhibited selective *lacZ* activation, while three showed *w*-specific activation by NEE. The remaining lines showed no activation of either *w* or *lacZ* by NEE. The 5′ *hairy* stripe one enhancer (HI) (Riddihough and Ish-Horowicz, 1991) is not affected by *Fab-7* or the PTS in most transgenic strains and is used as an internal control for enhancer strength. The pattern of promoter activation by NEE among different strains is similar to Type I, Type II, and Type III strains obtained when the PTS and *Fab-8* were included in a similarly constructed transgene. Thus, this result suggests that the 3.7 kb DNA may contain a new PTS element.

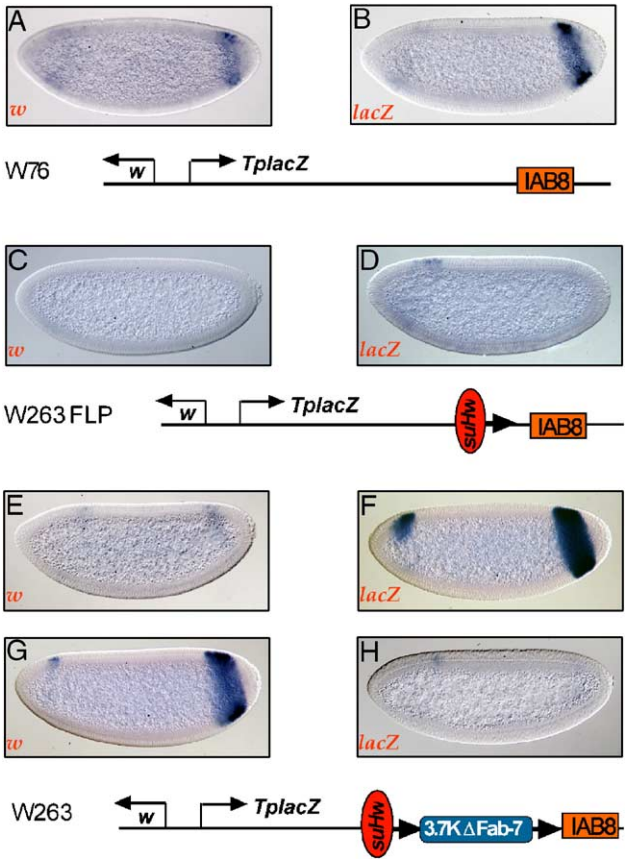

Fig. 3. Characterization of the promoter targeting activity from the 3.7 kb *Fab-7* region. (A, B) The IAB8 enhancer located 3′ of *lacZ* in embryos carrying the control transgene W76 activates both *w* and *lacZ* in low but detectable levels. (C, D) When the *suHw* insulator is present, IAB8 is blocked, and no transcription can be detected for either *w* or *lacZ*. This strain (W263FLP) was obtained from W263 (see below) after the 3.7kΔFab-7 is recombined away from the transgene. (E, F) When both *suHw* and 3.7kΔFab-7 are present, IAB8 activates robust *lacZ* expression but no *w* activation. (G, H) In a different strain, carrying W263, IAB8 selectively activates *w* instead of *lacZ*, leading strong staining in the posterior region of the embryo.

To definitively test the 3.7 kb region for promoter targeting activity, we tested this region against a heterologous insulator, *suHw*, in the #125 *P*-transformation vector described earlier (see Fig. 3) (Lin et al., 2003). This vector contains the 1.6 kb IAB8 enhancer located 3′ of the *lacZ* gene and a 360 bp *suHw* insulator (Cai and Levine, 1995) inserted between the *lacZ* and IAB8. The IAB8 enhancer alone at the 3′ of *lacZ* activates both *w* and *lacZ* producing moderate, but clearly detectable, transgene expression (Figs. 3A, B). When *w* and *lacZ* expression from embryos carrying W263FLP (same as #125) was analyzed, no detectable IAB8 activity could be seen (Figs. 3C, D). We then deleted the 0.8 kb *Fab-7* insulator from the 3.7 kb DNA and inserted the rest between the *suHw* insulator and IAB8. As shown in Figs. 3E through H, this *3.7ΔFab-7* is able to target the IAB8 enhancer to the *lacZ* promoter, overcoming the intervening *suHw* insulator. A total of 13 transgenic lines were analyzed, two of which targeted *lacZ* (type I strains, Figs. 3E, F), one targeted *w* (type II strains, Figs. 3G, H), while the remaining nine showed no promoter targeting: neither of the promoters is activated (data not shown, type III). To prove that

the IAB8 enhancer activity is due to promoter targeting rather than position effects associated with differential insertion sites, the two Type II transgenic strains were analyzed by FLP-induced recombination, which removes the intervening *3.7ΔFab-7* DNA flanked by the direct repeat of FRT sites (black arrows). Deleting the *3.7ΔFab-7* DNA from these transgenic strains leads to the total loss of IAB8 activity (Figs. 3C, D), suggesting that a PTS element exists within the 2.9 kb *3.7ΔFab-7*.

Mapping the minimal PTS

To map the minimal PTS, the 3.7 kb DNA was cut into three overlapping pieces, *F3.7a*, *F3.7b*, and *F3.7c* (Fig. 4A), and tested in transgenic vector #125. The result was summarized in Table 1. Approximately one third of the transgenic strains from *F3.7a* and a quarter from *F3.7b* exhibit promoter targeting. None of the transgenic line carrying *F3.7c* showed any strong (typical of promoter targeting) activation of either *w* or *lacZ* (Table 1), suggesting that the promoter targeting activity resides within *F3.7a* and *F3.7b*. Since there is only a 400 bp overlap between these two fragments, we tested the 200 bp DNA from the 3′ end of *F3.7a* and found that it is sufficient to mediate anti-insulator and promoter targeting activity (Table 1). This new 200 bp PTS is located just 230 bp 3′ of the *Fab-7* insulator, a similar position where the original PTS is located relative to *Fab-8*. To distinguish between the two PTS elements, we refer the newly discovered PTS as PTS-6 to reflect the fact that it is located in the *iab-6* domain. Similarly, we name the previously identified PTS as PTS-7.

Table 1
Summary of transgenic strains carrying DNA from the *Fab-7* region

Mapping the minimal PTS6

construct	test DNA	*w*	*lacZ*	no T	total
W205	F3.7 a	4	3	11	18
W206	F3.7 b	2	4	13	21*
W204	F3.7c	0	0	12	14*
W208	PTS–6	2	3	5	12*
W263	3.7K ΔFab-7	2	2	9	13

These DNA sequences were inserted between the IAB8 enhancer and the *suHw* insulator located at the 3′ of *lacZ*. Transformants were classified into three types according which promoter is activated by IAB8 (Lin et al., 2004). Briefly, in Type I, IAB8 activates *lacZ*, not *w*. In Type II, it activates *w* but not *lacZ*, while, in Type III, the IAB8 enhancer activates neither *w* nor *lacZ*. The selective activation of a single promoter is an indication of promoter targeting activity. Asterisks indicate that several strains obtained exhibit excessive enhancer trap or background staining that prevents them from being characterized as one of the three categories.

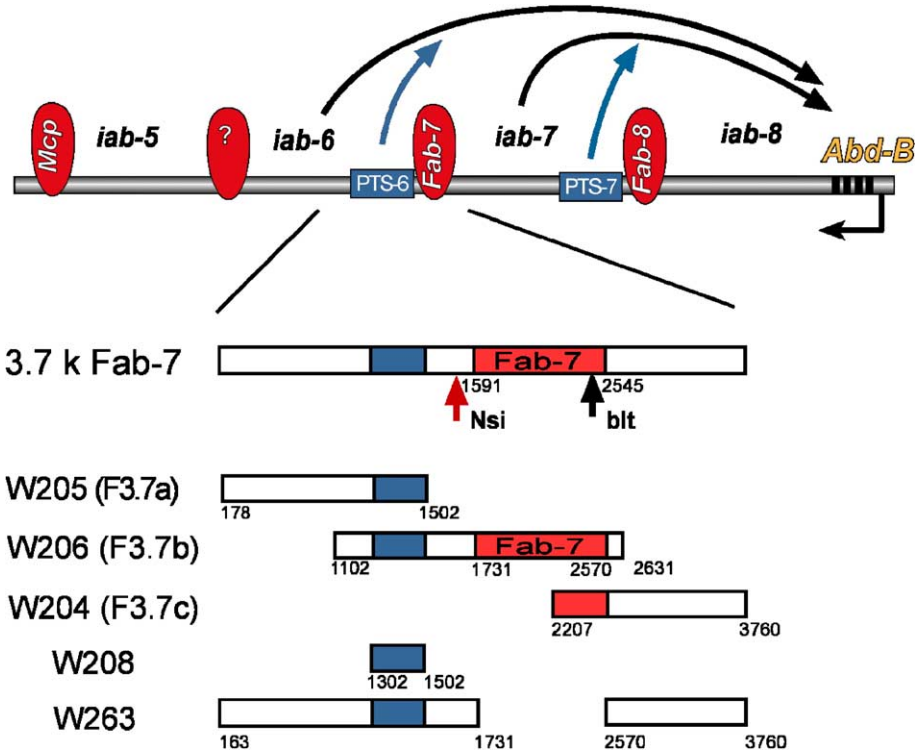

Fig. 4. Mapping the PTS-6 element from the 3.7kb *Fab-7* region. Diagram showing the different regions tested from the 3.7 kb *Fab-7* region. Top diagram shows the structure of the *Abd-B* locus. The promoter (arrow) and coding regions (black bars) are located to the far right. The *iab-9* region located upstream is not shown. The 3′ regulatory regions, *iab-5* through *iab-8*, are shown. Red ovals represent domain boundaries. Blue squares indicate Promoter Targeting Sequences.

During our analysis, we noticed that, when NEE is targeted by PTS-6 or PTS-7, it is only moderately facilitated (see Fig. 2, compare B with F). However, when IAB8 is targeted, a much greater degree of facilitation is observed (see Fig. 3, compare B with F and G). This result suggests that PTS elements may be more efficient in facilitating *Abd-B* enhancers than heterologous enhancers. Supporting this notion, we found that PTS-7 provides very little augmentation to the *even skipped* stripe 3 enhancer (Small et al., 1993) (data not shown), while it greatly enhanced the IAB5 enhancer (Lin et al., 2003).

PTS elements overcome multiple insulators

Unlike enhancers from the *iab-7* domain, which are separated from the *Abd-B* promoter by a single insulator, *Fab-8*, enhancers from *iab-6* must overcome two insulators, *Fab-7* and *Fab-8*. To test whether PTS-6 could overcome both of these insulators, we inserted the *Fab-7*, *Fab-8*, and PTS-6 between the 3′ of *lacZ* and the IAB8 enhancer (see W270 in Fig. 5). After transgenic strains were analyzed by in situ hybridization, we detected all three types of transgenic strains: 5 of the 21 strains target *lacZ*, while three selectively activate *w*. A representative line showing *lacZ* targeting is shown in Figs. 5C and D. In the control experiment, the 0.8 kb *Fab-7* and the 0.7 kb *Fab-8* were inserted between the 3′ of *lacZ* and the more 3′ IAB8. No *w* or *lacZ* activation could be detected after analyzing 6 transgenic strains carrying this construct, suggesting that IAB8 is blocked (Figs. 5A, B). These results suggest that, in principle, PTS-6 may be able to target an enhancer over both *Fab-7* and *Fab-8* insulators in the endogenous *Abd-B* locus.

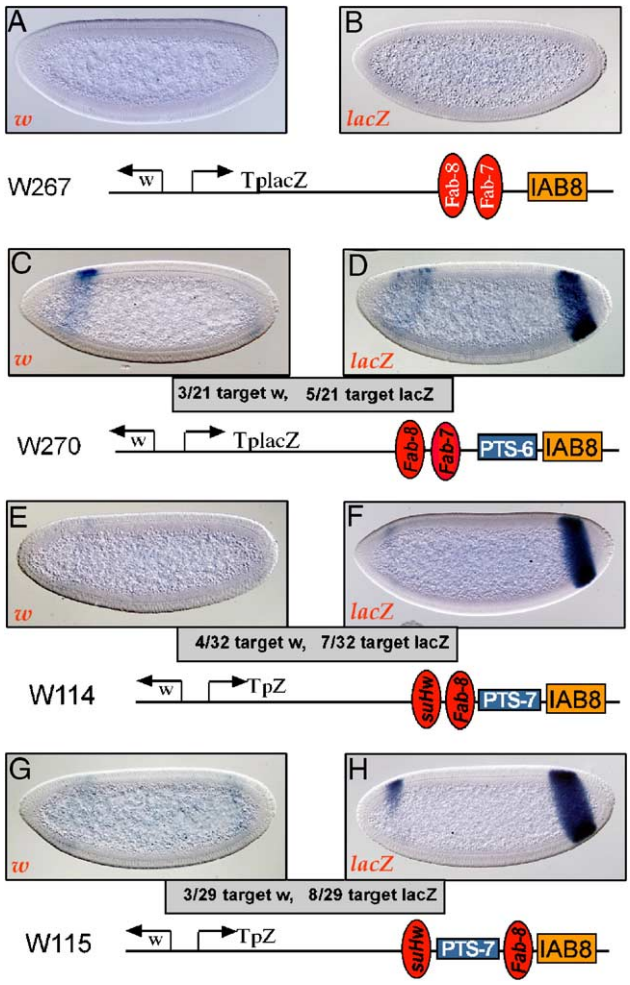

Fig. 5. The PTS element overcomes multiple insulators from different positions. (A, B). The combination of the 0.8 kb *Fab-7* and 690 bp *Fab-8* insulators totally blocks the IAB8 enhancer. No transgene expression can be detected. (C, D) The 200 bp PTS-6 can overcome *Fab-7* plus *Fab-8* insulator combination and selectively activates *lacZ* (in the strain shown). From 21 strains analyzed, three strains selectively activated *w*, five selectively activated *lacZ*, and the rest did not show activation of either promoters. (E, F) The PTS-7 element overcomes a combination of *suHw* and *Fab-8* and selectively activates *w* in 4 of 32 lines or *lacZ* in 7 of 32 lines. (G, H) When PTS-7 is placed between *suHw* and *Fab-8*, it targets IAB8 to *w* in 3 of 29 strains and to *lacZ* in 8 of 29 lines analyzed.

To test whether overcoming multiple insulators is a general property of PTS elements, we also challenged PTS-7 with two insulators, *Fab-8* and *suHw*. This experiment was done by inserting the 1.7 kb region (Zhou and Levine, 1999) from *Fab-8* (containing both the *Fab-8* insulator and the 625 PTS-7) into #125 (see construct W114 in Fig. 5, under E, F). Similar to PTS-6, PTS-7 overcomes a combination of two insulators, *suHw* and *Fab-8*, since IAB8 strongly activates *lacZ* in seven of 32 strains tested, and it activates *w* in four of these lines. An example of *lacZ*-targeted strain is shown in Figs. 5E, F, where IAB8 activates only *lacZ*. There is no detectable level of *w* expression. Similar results were obtained when the 3.7 kb DNA containing both PTS-6 and *Fab-7* was inserted between *suHw* insulator and IAB8 enhancer in transgenic vector #125 (data not shown). These data suggest that there is no difference between PTS-6 and PTS-7 in terms of bypassing which insulator or insulator combination, thus overcoming that multiple insulators is a general property of the PTS.

Previous genetic analyses suggested that, when both PTS-7 and PTS-6 are deleted from *Abd-B* locus, none of the enhancers from *iab-5, iab-6*, or *iab-7* region could activate *Abd-B* (Zhou and Levine, 1999). This implies that PTS-6 and PTS-7 might play a role in targeting enhancers from the *iab-5* region. To test if this may be possible, we constructed a transgenic vector that partly mimics the arrangement of different *cis* elements in the *iab-5* and *iab-6* region relative to the *Abd-B* promoter. As illustrated in W115 of Fig. 5, PTS-7 is located at the 3′ end of *lacZ*, flanked by a pair of insulators, *suHw* and *Fab-8*. The IAB8 enhancer is placed at the most 3′ position. This layout of different *cis* elements in the transgene is similar to the endogenous arrangement of *cis* elements in *Abd-B* in that there are insulators both between the PTS and the promoter and between the PTS and the enhancer. When transgenic strains carrying this construct were analyzed, we could readily recover Type I (8/29), Type II (3/29), and Type III (18/29) strains, indicating that the PTS medicates promoter targeting in this transgene. One example of Type I strains is shown in Figs. 5G, H. Here, IAB8 strongly activates *lacZ*, while the neighboring *w* promoter is not activated. This result suggests that the PTS is able to mediate promoter targeting from various positions relative to the insulator, thus

in the endogenous location PTS-6 (as well as PTS-7) could potentially regulate *iab-5* enhancers.

Discussion

Here, we described the identification and characterization of a new Promoter Targeting Sequence, PTS-6, from the *iab-6* domain of the *Abd-B* 3′ regulatory region. PTS-6 is located just next to the *Fab-7* boundary. It overcomes both the *Fab-7* insulator and the heterologous *suHw* insulator and selectively targets enhancers to a single promoter in transgenic embryos. PTS-6 preferentially facilitates the IAB8 enhancer from *Abd-B* to bypass the heterologous NEE enhancer. More importantly, PTS elements could overcome a combination of two insulators including *Fab-7* plus *Fab-8* and could function from a number of positions relative to the insulators. These findings suggest that PTS elements could target distal enhancers in *Abd-B* over several insulator elements to activate the promoter. These results strongly support the promoter targeting model of long-range enhancer–promoter interactions in the *Abd-B* locus.

The function of the PTS-6 is consistent with our previous genetic study (Zhou and Levine, 1999), where a *P*-element insertion into the PTS-7 region produced loss of function of *Abd-B* only in the 7th abdominal segment. However, a deletion in PTS-7 in combination with the deletion of 3.7 kb in $Fab\text{-}7^{1\ R73}$ (which removes both the *Fab-7* insulator and the PTS-6) produced a much stronger loss of function phenotype, which is evident as almost a complete loss of *Abd-B* function in A5, A6, and A7 segments (Zhou and Levine, 1999). Although the deletion of PTS-7 alone is not available, this result, nonetheless, suggests that the deletion of PTS-6 (plus *Fab-7*) enhances the loss of function phenotype of PTS-7 mutation. Since the $Fab\text{-}7^{1}$ mutation alone does not have loss of *Abd-B* function phenotype in A5 and A6 abdominal segments (Gyurkovics et al., 1990; Mihaly et al., 1997), PTS-6 and PTS-7 may have redundant functions.

Our study provides support to the model that the *iab* regulatory regions in *Abd-B* have modular organizations near the domain boundary, i.e. PTS, insulator, and PRE elements could be found near the boundary region. Although the *iab-5/iab-6* boundary has not been studied, this is apparently the case for both *iab-6/iab-7* and *iab-7/iab-8* boundaries. In both cases, these elements are arranged in the same order: PTS–insulator–PRE. It is not clear why the PTS elements are located so close to the insulators in both cases (less than 300 bp). In transgenic embryos, separating the PTS from the insulator by 3 kb λ insertion (Lin et al., 2003) and a 6 kb λ insertion (Zhou J., unpublished results) did not affect promoter targeting. However, in the endogenous location, this arrangement could be of functional significance. For example, the close proximity between the insulator and the PTS may be necessary for the protection of the "enhancer–promoter complex" against the PRE element located just opposite of the insulator when an *iab* domain is looped to the promoter.

In this study, PTS-6 overcomes both the homologous *Fab-7* insulator and the heterologous *suHw* insulator. In addition, it overcomes a combination of both *suHw* and *Fab-7*. These results are in apparent disagreement with a previous study where the replacement of the endogenous *Fab-7* insulator with *suHw* led to the loss of *Abd-B* function in the 5th and 6th abdominal segments (Hogga et al., 2001). One of the interpretations was that the *suHw* is a stronger insulator than *Fab-7*, consequently, enhancers located in *iab-5* and *iab-6* are blocked and could not activate sufficient *Abd-B* expression. Results from our current study suggest that the strength of an insulator does not seem to affect the promoter targeting function of the PTS: the PTS facilitates the IAB8 enhancer equally well, regardless of which insulator, or insulators, are in its way. In light of our findings, we favor the alternative interpretation of the insulator replacement study, which emphasizes the qualitative differences between *Fab-7* and other heterologous insulators. For example, *Fab-7* has been shown to be developmentally regulated, while *suHw* is not (Schweinsberg and Schedl, 2004).

Previous observations with the *suHw* insulator suggested that two copies of the insulator could interact and cancel the enhancer blocking function (Cai and Shen, 2001; Muravyova et al., 2001). In our study, we also tested several combinations of insulators: *Fab-7/Fab-8*, *suHw/Fab-8*, and *Fab-7/suHw* (Chen Q. and Zhou J., unpublished results). In all three combinations, the double insulators exert stronger enhancer blocking than a single one. Thus, these insulators do not appear to interact with each other to cancel enhancer-blocking function as do two *suHw* insulators.

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